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SUBJECT OF INVESTIGATION

BIOCHEMICAL AND GENETICAL STUDIES ON INH METABOLISM

RESPONSIBLE INVESTIGATOR

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BIOCHEMICAL AND GENETICAL STUDIES ON INH METABOLISM

INTERIM REPORT

DA-CRD-AG-S92-544-63-G3

30 September 1963

Shigeichi Sunahara

Tokyo National Chest Hospital

CONTENT

BIOCHEMICAL AND GENETICAL STUDIES ON INH METABOLISM

I. CONFIRMATION OF THE TRIMODALITY OF THE FREQUENCY DISTRIBUTION CURVE OF THE BIOLOGICALLY ACTIVE ISONIAZID LEVELS IN SERA OF CAUCASIANS

Sunahara-Urano's hypothesis about the genetical patterns of isoniazid inactivation is based on their observation of the trimodal frequency distribution curve of the biologically active isoniazid concentrations 6 hours after the dose of 4 mg/kg body weight of isoniazid corresponding to rapid, intermediate and slow inactivation of isoniazid in the human body and the validity of their hypothesis has been verified both by the family study and by the population survey on the Japanese, Ainus, Koreans, Chinese and Thai. (c f. Annual Report for 1962, Science 134, 1530, 1961 and Bulletin of the International Union against Tuberculosis 32, 513, 1962)

According to their hypothesis, slow and rapid inactivators are homozygous and intermediate heterozygous, and inactivation of isoniazid is concerned with inheritance without dominance. But other investigators, such as Knight and Harris, Evans and McKusick, etc. were failed to establish a trimodal distribution curve and they were only successful in distinguishing two modes: "slow" and "rapid". Though Knight et al mentioned "intermediate" too, this term meant for them nothing but an antimode or a valley in the trimodal distribution curve, while "intermediate" in our classification is a mode or a distinctly defined group of subjects quite similar to "slow" and "rapid". It is the reason why American and English researchers hesitate to approve Sunahara's genetical hypothesis thoroughly.

The cause of the discordance between the distribution curve in the Japanese reported by Sunahara et al and that in the Caucasians (or negroes) reported by Knight, Evans, etc. has not been cleared up yet, but as it is illogical to suppose that a different law of inheritance holds for the Japanese and Caucasians, the difference in laboratory techniques used by Sunahara and other investigators must play an important role. For instance, Clark discussed: "Sunahara et al (1960) have shown, using a very refined microbiological technique that there is a trimodal distribution of serum isoniazid in Japanese patients. This finding suggests that their technique is able to separate out the three genotypes present in the population but this has not been conclusively proved by means of an appropriate family studies." (C.A. Clark: "Genetics for the Clinician", Oxford, 1962, p.191) (The latter part of his comment is concerned with our earliest report, for we are now successful in testing our genetical hypothesis based on the result of family study as indicated in the annual report 1961 and other reports of ours.)

We established trimodal distribution curve in the Chinese and the Thai, a larger percentage of which are slow inactivators as in the Caucasians, but we have not been successful in collecting a sufficient number of blood of the Caucasians to draw a distribution curve.

Early in 1963, Knight and Harris and we agreed to exchange sera of the Caucasians and the Japanese between Philadelphia and Tokyo. Knight et al determined active isoniazid concentration by means of the vertical diffusion method modified by Mitchison which is a little different from ours in detail. For instance, agar solution is added to serum and drug is diluted by distilled water with agar instead of serum for the preparation of a standard curve, etc.

Fig. 1 & 2 show the frequency distribution curve for the Caucasians prepared by Knight et al and by us respectively. Based on this investigation, Knight wrote me recently: "We have confirmed your finding of trimodality of the frequency distribution curve in Caucasians and Japanese population...The difference in technique probably accounts for the small difference between your result and ours..."

At last we are successful in establishing trimodality of frequency distribution curve of the Caucasians and consequently the validity of our genetical hypothesis will be verified, we believe, thoroughly regardless of kinds of human races.

Fig 3 indicates the frequency distribution curve for the Japanese drawn based on the data reported by Knight et al.

Fig. 1 Frequency distribution curve of Caucasians (108 subjects) determined by a modified vertical diffusion method —Knight et al 1963

Fig. 2 Frequency distribution curve of Caucasians (108 subjects) (same sera as in Fig 1) determined by Urano and Sunahara

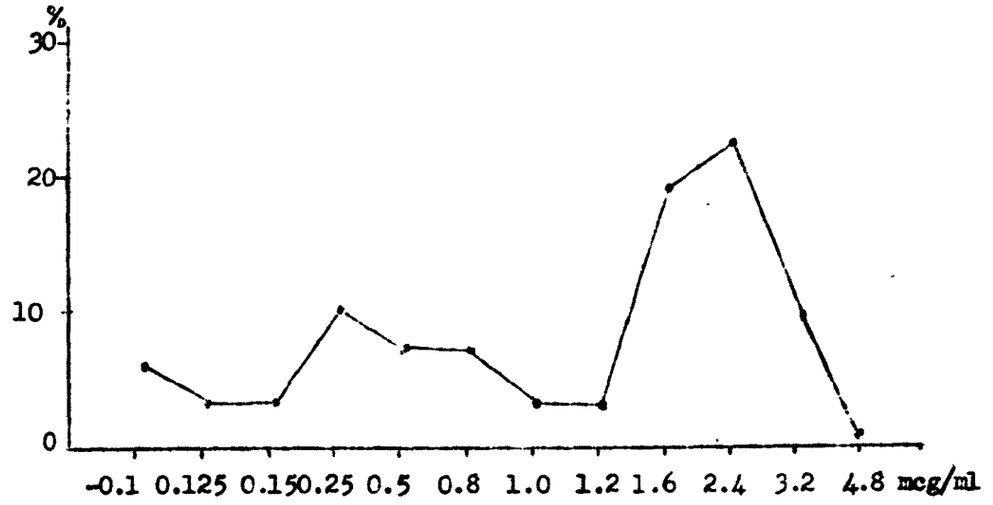
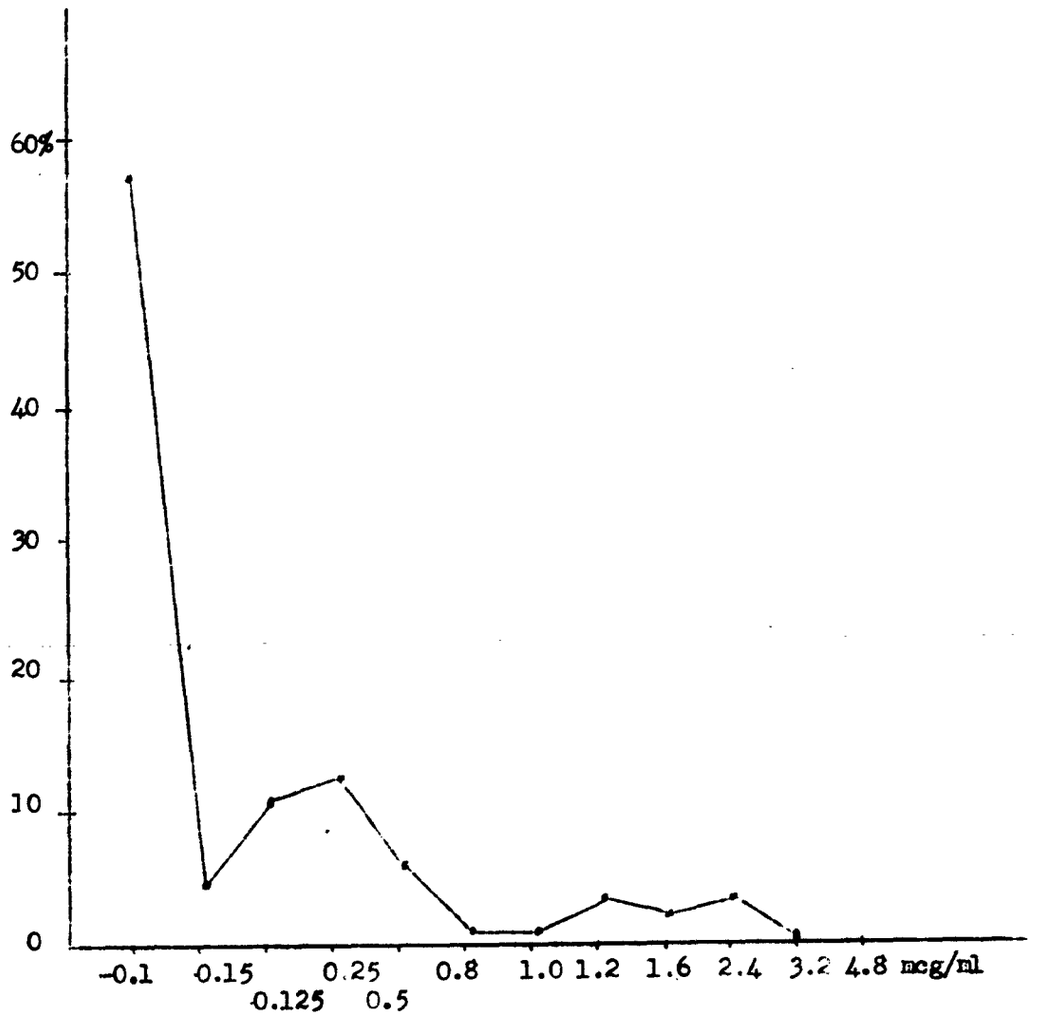


Fig. 3 Frequency distribution curve of Japanese (105 subjects) by means of a modified vertical diffusion method—Knight et al 1963



II. BIOCHEMICAL STUDIES ON ACETYLATION IN ANIMAL TISSUE

Not only species difference but also drug difference in respect to acetylation in animal tissue was observed by us as reported already. For instance, isoniazid was inactivated very vigorously by chicken liver or pigeon liver, while it was only slightly affected by rabbit or rat liver. Acetylation of sulfisoxazole and PABA was very weak in chicken liver, while sulfonilamide was inactivated in chicken and pigeon liver quite markedly. PABA was acetylated very actively only by pigeon liver. We reported also that the addition of acetate, pyruvate or co-A to the reaction system enhanced acetylation to a remarkable degree in the chicken or pigeon, while slight increase in acetylation was recorded in the guneapig or rat.

1. EFFECT OF ATP ON ACETYLATION

ATP is an essential energy source for acetylation as Lipmann demonstrated. Using Warburg vessel, we studied the relationship between the amount of ATP and acetylating activity of chicken liver homogenate. The experimental condition was as follows:

Main chamber	1/15 M phosphate buffer	0.3 ml	
	0.1 M mg cl	0.1	
	0.4 M Na F	0.1	Temperature: 37°
	0.1 M Na acetate	0.4	Gas phase: Air
	500 mcg/ml INH	0.4	Incubation time: 30 min.
	0.4 g homogenate	1.0	
	H_2O	0.4	
Side chamber	20% KOH	0.2	

As indicated in Fig. 4, though augmentation in the rate of acetylation is observed after the addition of ATP to the reaction system, it is difficult to exceed 50% regardless of the increased doses of ATP. The endogenous ATP in liver tissue accounts for 16% acetylation without any additional ATP.

Fig. 5 illustrates that there is a close correlation between acetylation of sulfonilamide by the acetone powder extract of chicken liver and the amount of 0.15 M ATP added to a Kaplan-Lipmann's reaction system.

Fractional determination of adenosine derivatives being difficult, we measured Δ7P content of the liver of several kinds of animals as shown in Fig. 6. It is needless to note that ADP, AMP and creatin phosphate were also measured as Δ7P but ATP was believed to account for 70-80% of Δ7P. As Fig. 6 shows there is almost no difference in average content of Δ7P of the liver of various animals, it is quite natural to suppose that the

species difference in acetylating activity is not due to ATP content of liver tissue. The pigeon liver which has the most actively acetylating capacity is almost equal to the rat or mouse liver which acetylates isoniazid to the least degree with respect to the ATP content.

2. RELATIONSHIP BETWEEN CO-A AND SPECIES DIFFERENCE IN ACETYLATION

In the Annual Report 1962, we demonstrated that there was no correlation between co-A content of the liver of several species of animals and their capacity to acetylate isoniazid, sulfonilamide or PABA.

Fig. 7 represents that the acetylating activity increases to a certain extent when co-A is added to acetone powder extract of pigeon liver, but it reaches the maximum limit for each drug, sooner or later. The order of the capacity is PABA, INH and sulfonilamide. In Fig. 7/A , a diagram is reproduced from the Annual Report 1962 to indicate the comparative capacity of pigeon liver homogenate to acetylate isoniazid, sulfonilamide and PABA.

Fig. 8 deals with acetylation of the drugs by acetone powder extract of chicken liver. It is noteworthy that acetylation of PABA and sulfonilamide remained almost unchanged even after the addition of the maximum amount of co-A to the reaction mixture,
while isoniazid is acetylated markedly. Comparison of the rates of acetylation among these three kinds of drugs by chicken liver homogenate is shown in Fig. 8/A.

The relationship between drug and species difference in acetylation by acetone powder extract is illustrated in Fig. 9. Isoniazid is acetylated very vigorously by the pigeon and also by the chicken but only to a negligible degree by the guineapig regardless of an increasing amount of co-A added to the reaction system. It is remarkable that chicken liver shows very weak capacity of acetylation in sharp contrast to pigeon liver.

Based on these observations, the tentative conclusion has been reached that the species difference in acetylation does not depend on the content of ATP, co-A or certain substrates in animal tissue but on either quantitative or qualitative difference in acetylase found in various kinds of animals.

According to Lipmann, the stages of acetylation are understood as follows:

(1) Acetate + co-A $\xrightarrow[\text{Acetylkinase}]{\text{ATP}}$ Acetyl co-A

(2) Acetyl co-A + Drug $\xrightarrow[\text{Transacetylase}]{}$ Acetylated drug + co-A

Our further research will be concerned with differentiation of acetylkinase and transacetylase and approach to the mechanism of individual, species and drug difference in acetylation.

Fig. 4 Effect of ATP added to the reaction system on acetylation of INH by chicken liver homogenate

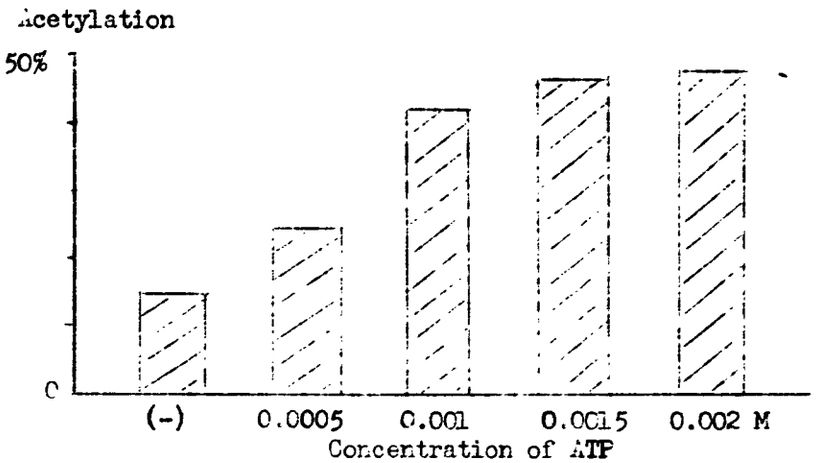


Fig. 5 Relationship between acetylation of sulfonilamide by acetone powder extract of chicken liver and amount of 0.15 M ATP added to the reaction mixture

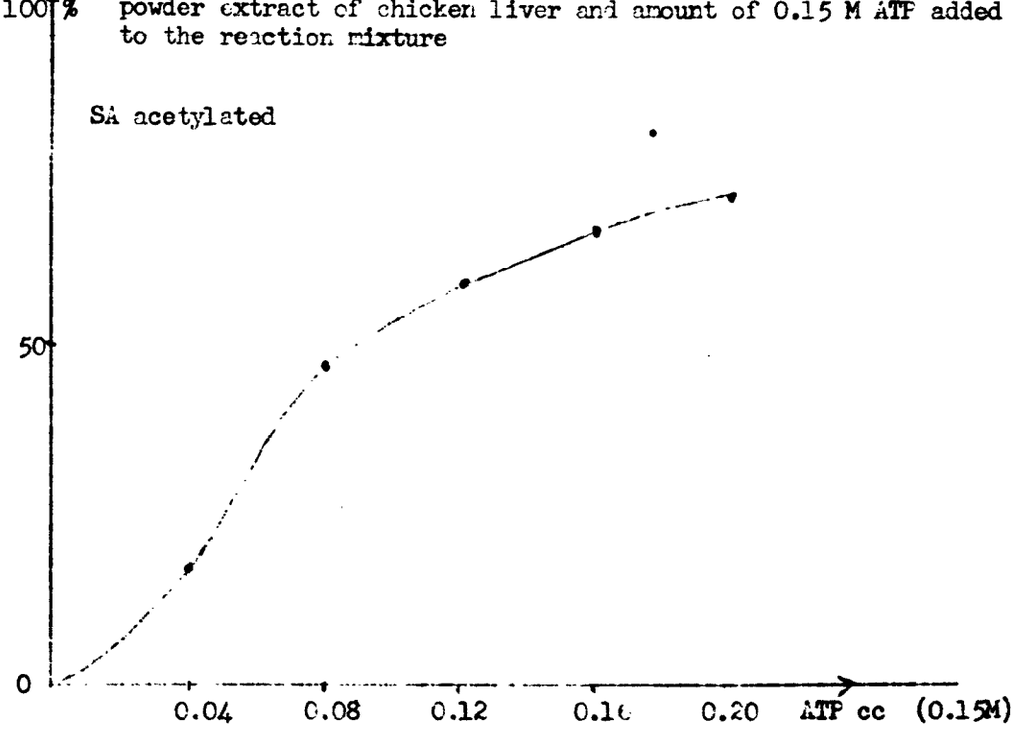


Fig 6 ATP content of the liver of various kinds of animals

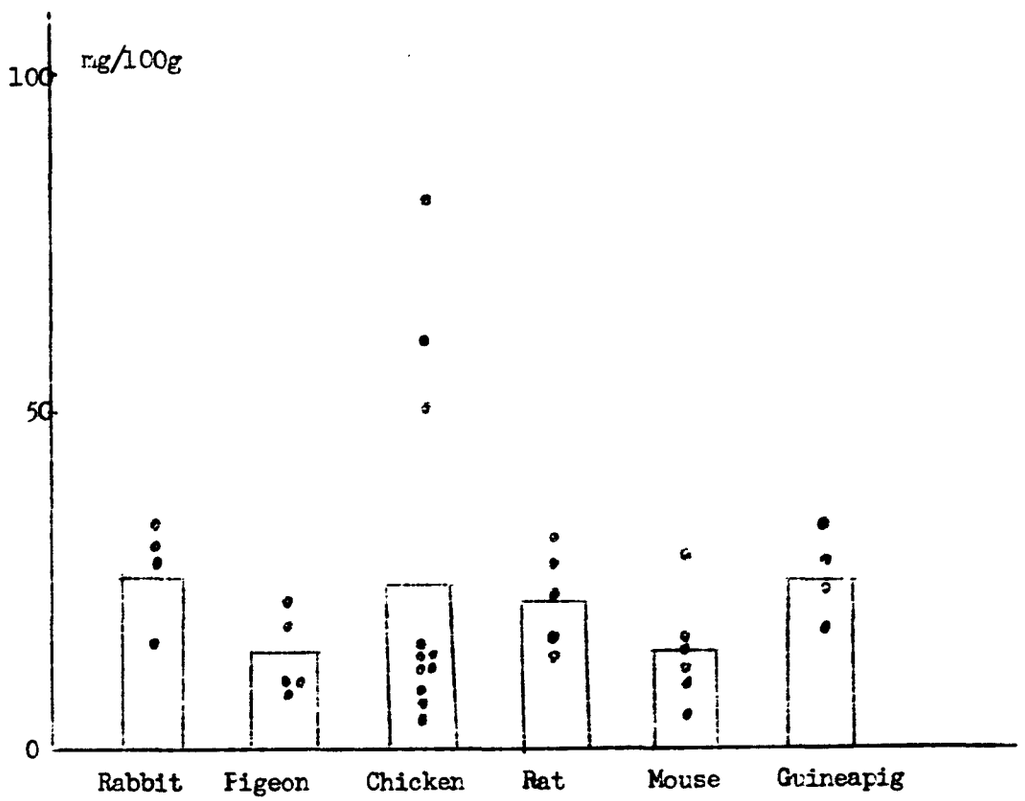


Fig 6 ATP content of the liver of various kinds of animals

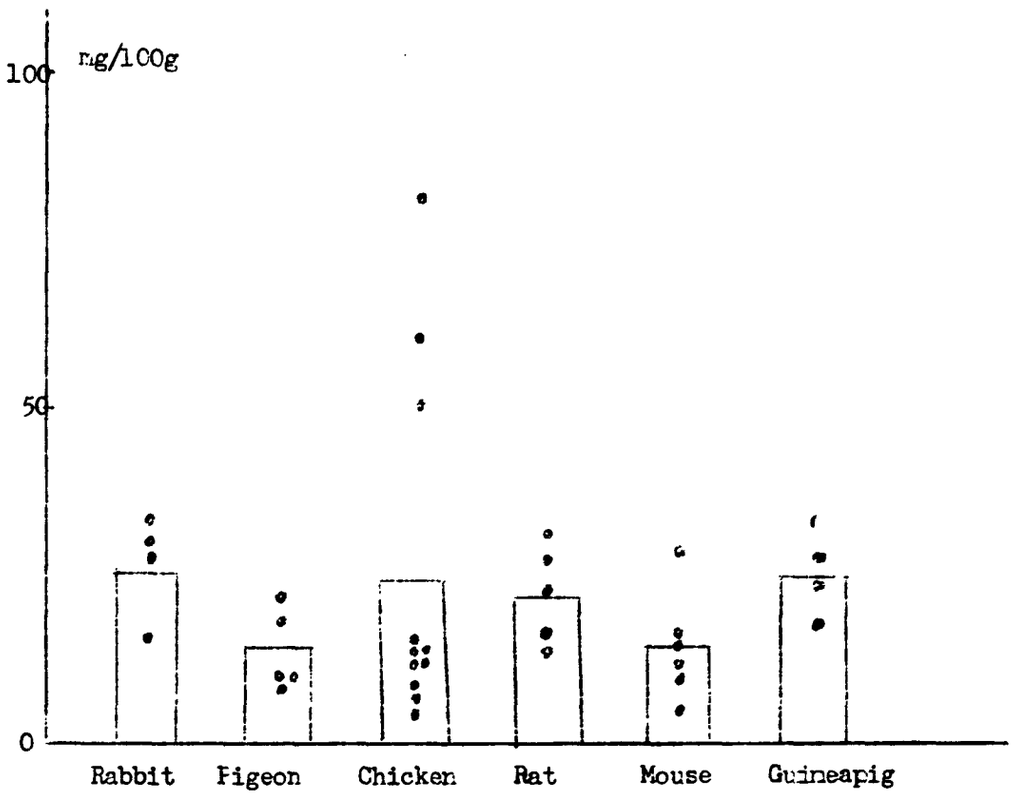


Fig. 7 Capacity of aceton powder extract of pigeon liver to acetylate PABA, INH and sulfonilamide, related to a dose of co-A to reaction system

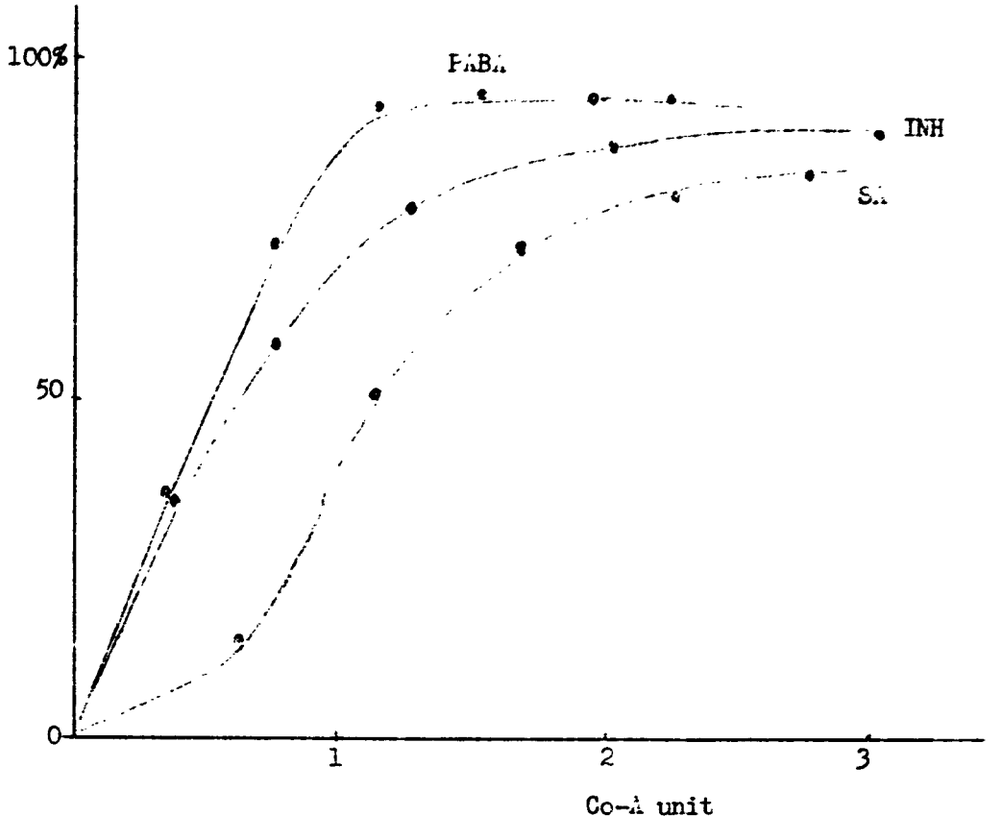


Fig. 7/A Acetylation by pigeon liver homogenate

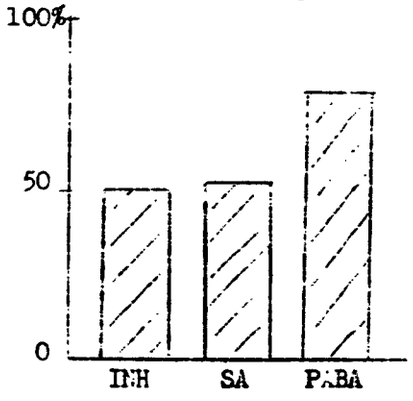


Fig. 8 Capacity of acetone powder extract of chicken liver to acetylate INH, sulfonilamide and PABA, related to a dose of co-A added to reaction system

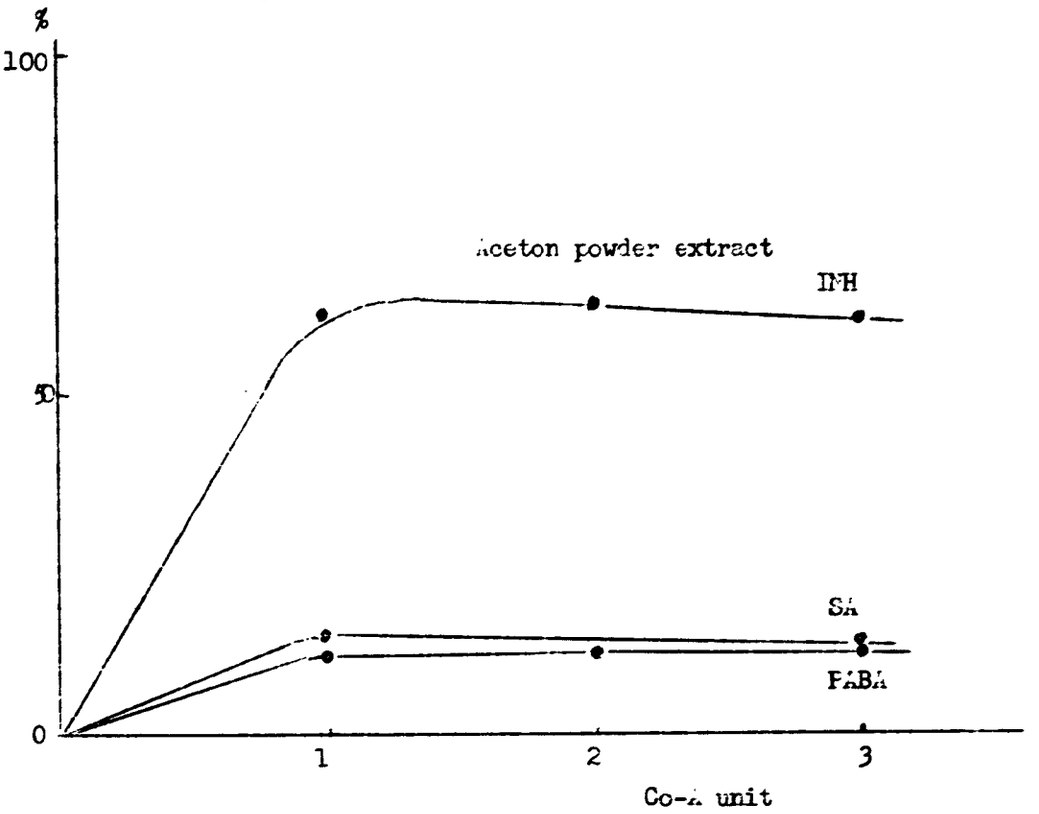


Fig. 8/A Acetylation by chicken liver homogenate

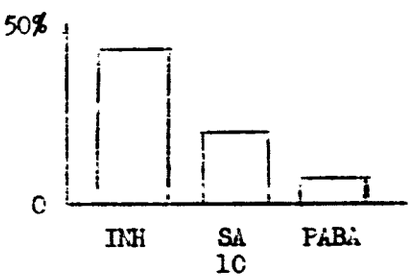
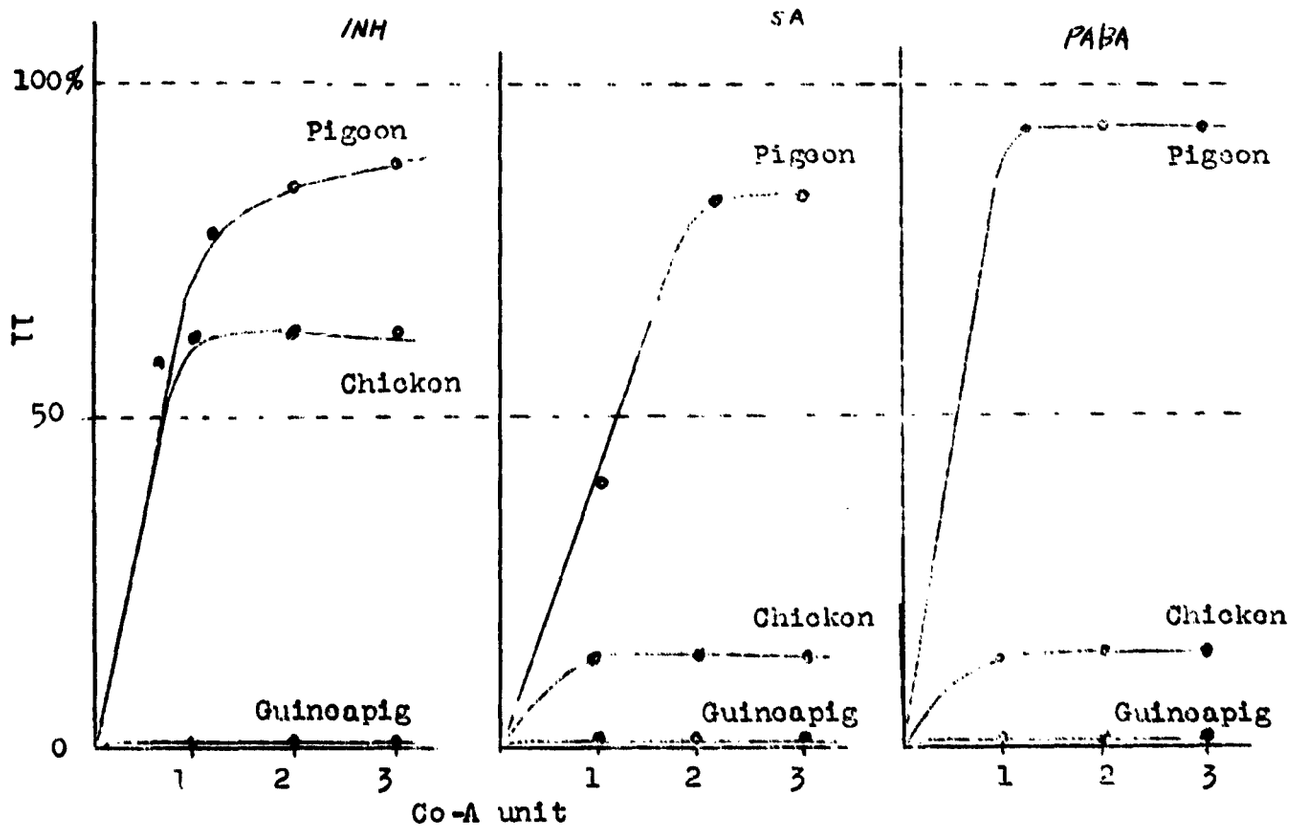


Fig. 9 Species and drug difference in acetylation



III. DETERMINATION OF ISONIAZID DERIVATIVES EXCRETED IN URINE BY A NEW METHOD

We have studied urinary excretion of metabolites of isoniazid by means of Hughes' method which is a modification of Short's method. As Short published recently a new modification of his method (Tubercle 42, 218-226, 1961), we tried to compare this method with Hughes' method.

In our previous study, we measured free isoniazid and hydrazone by means of Short's method modified by Hughes, and acetyl isoniazid by Kelly-Foet's technique modified by Tayama. In the present study, Short's technique which depends on the reaction of 1:2 naphthoquinone-4-sulphonic acid with the two hydrogen atoms of the $-NH_2$ group of hydrazid was used for the determination of free isoniazid, hydrazone and acetyl isoniazid.

Table 1 shows the actual values of estimation by Hughes' method (A) and the new Short's technique (B). Table 2 & 3 indicate amounts and percentages of the three metabolites excreted in urine of three subjects after the oral dose of 4 mg/kg body weight calculated on the basis of the values in Table 1. The values for hydrazone by the new method is inferior to that by the old method and the exact converse is true with the value of acetyl isoniazid as indicated in Fig. 10.

The estimation of acetyl isoniazid, hydrazone isoniazid and free isoniazid on 15 subjects is charted in Fig. 11 & 12. The major part of acetyl isoniazid is excreted during the first 12 hours, while that of free isoniazid and hydrazone during the first 6 hours. The total amount of the three compounds accounts in average for 53.6% of the dose of the administration as shown in Table 4 and Fig. 13.

Fig. 14, 15 & 16 demonstrate the relationship between the biologically active isoniazid concentrations 6 hours after the dose of 4 mg/kg body weight of isoniazid on the one hand and acetyl isoniazid or free isoniazid in 6, 12 or 24 hour urine on the other.

The distinction of the three patterns of isoniazid inactivation based on urinary excretion of metabolites in Fig. 14 which is concerned with 6 hour is most clear-cut as compared with Fig. 15 and 16, but the number of the examined is too small to draw a definite conclusion that we are able to classify the three kinds of the genetical trait on the basis of urinalysis.

Table 1 Actual values for 3 hour urine (mg)

Method	Kinds of metabolites	Experiment No.1	No. 2	No. 3
A	F	7.4	7.8	4.5
	F+H	12.5	11.7	8.2
	F+H+A	42.0	44.5	45.3
B	F	7.0	7.7	4.1
	F+H	8.6	10.2	6.1
	F+H+A	41.0	39.7	46.5

Table 2 Amount of three kinds of metabolites in 3 hour urine (mg)

Method	Kinds of metabolites	Experiment No.1	No.2	No.3
A	Free INH	7.4	7.8	4.5
	Hydrazone INH	5.1	3.9	3.7
	Acetyl INH	29.5	32.8	37.1
B	Free INH	7.0	7.7	4.1
	Hydrazone INH	1.6	2.5	2.0
	Acetyl INH	32.4	29.5	40.4

Table 3 Relative amount of metabolites (%)

Method	Kinds of metabolites	Experiment No. 1	No. 2	No. 3	Average
A	Free INH	17.6	17.5	9.9	15.0
	Hydrazone INH	12.1	8.7	8.2	9.7
	Acetyl INH	70.3	73.8	81.9	75.3
B	Free INH	17.1	19.4	8.8	15.1
	Hydrazone INH	3.9	6.3	4.3	7.7
	Acetyl INH	79.0	74.3	86.9	80.2

Fig. 10

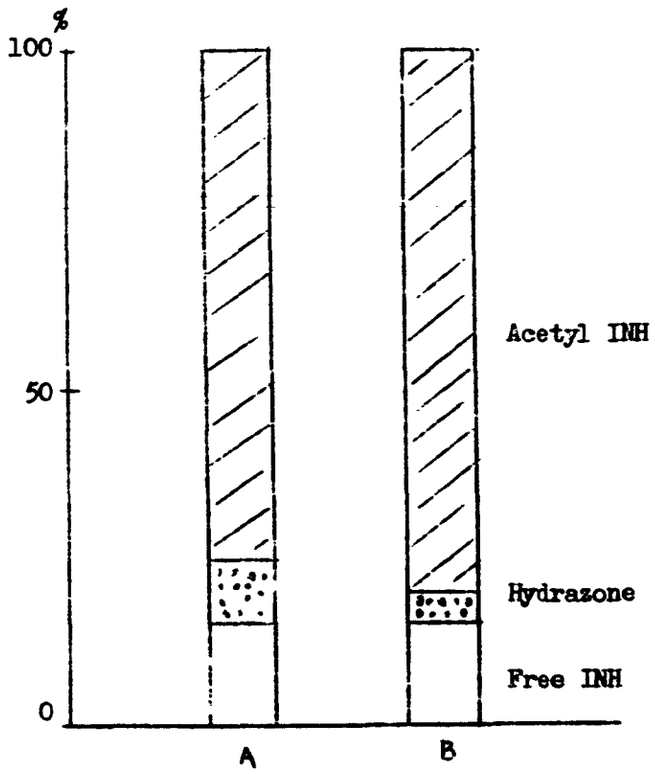


Fig. 11 Urinary excretion of acetyl, hydrazone and free INH in 15 cases after 4 mg/kg of oral administration of INH

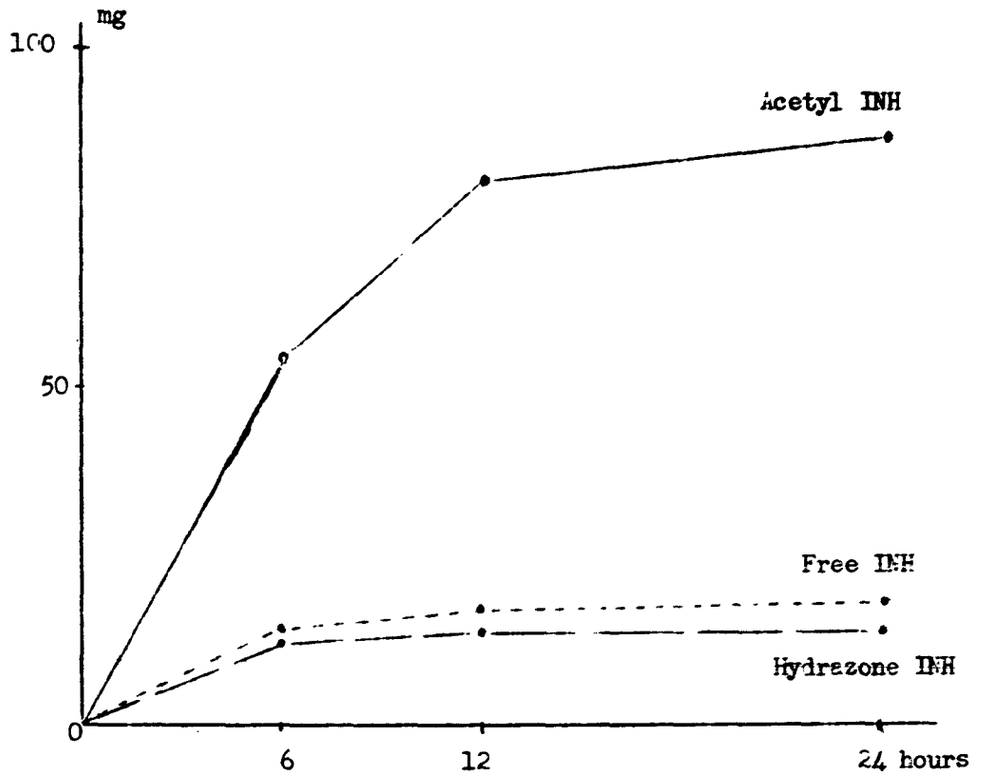


Fig. 12 Urinary excretion of acetyl, hydrazone and free INH in percentage (Average of 15 cases)

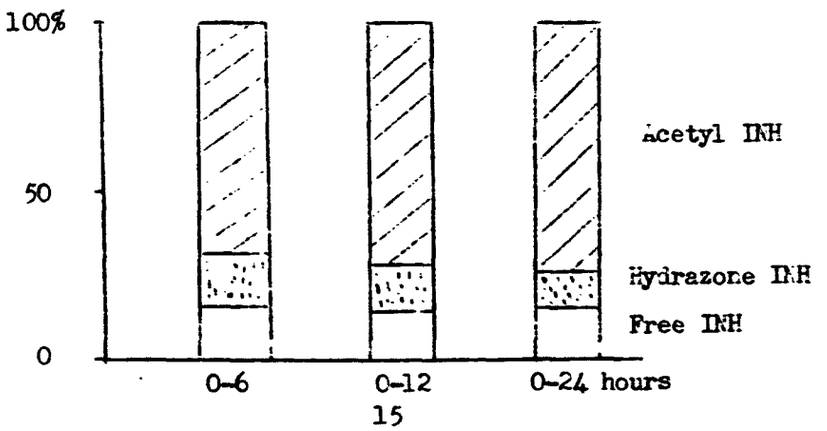


Table 4 Urinary excretion of total INH after 4 mg/kg of oral administration of INH in 15 cases

	in 6 hours	in 12 hours	in 24 hours
Average	37.6%	50.6%	56.3%
Minimum	16.9	25.2	34.2
Maximum	52.4	64.5	72.6

Fig. 13 Total INH excreted in urine

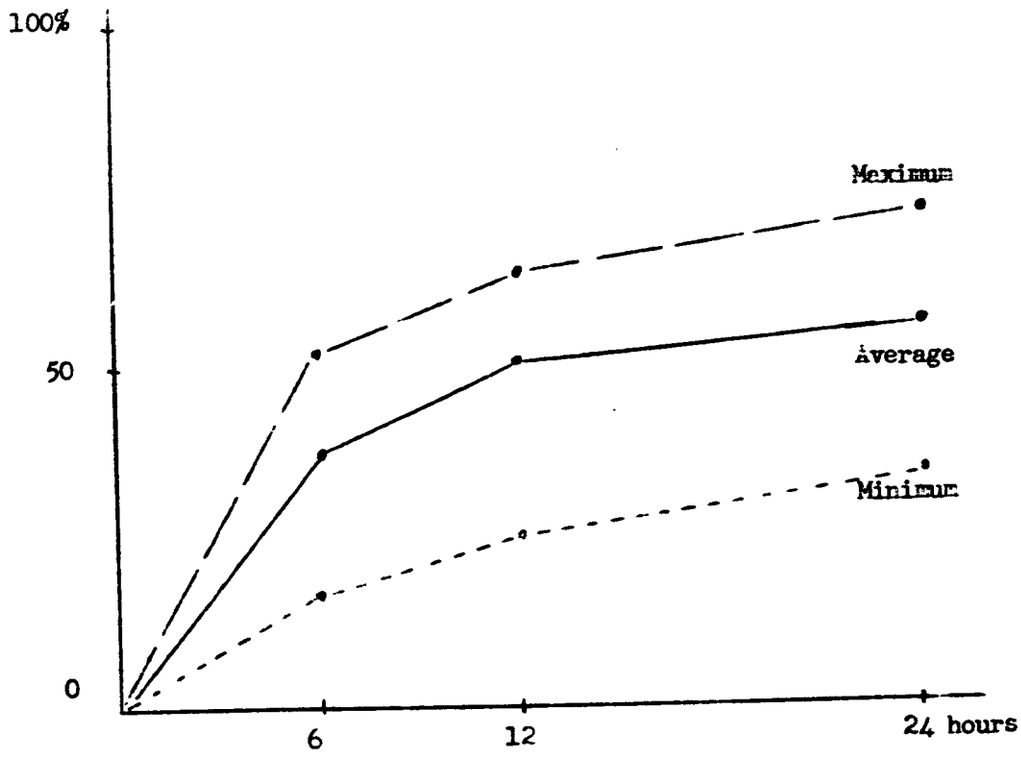


Fig. 14 Relationship between INH concentration in blood and acetyl and free INH in urine in 6 hours

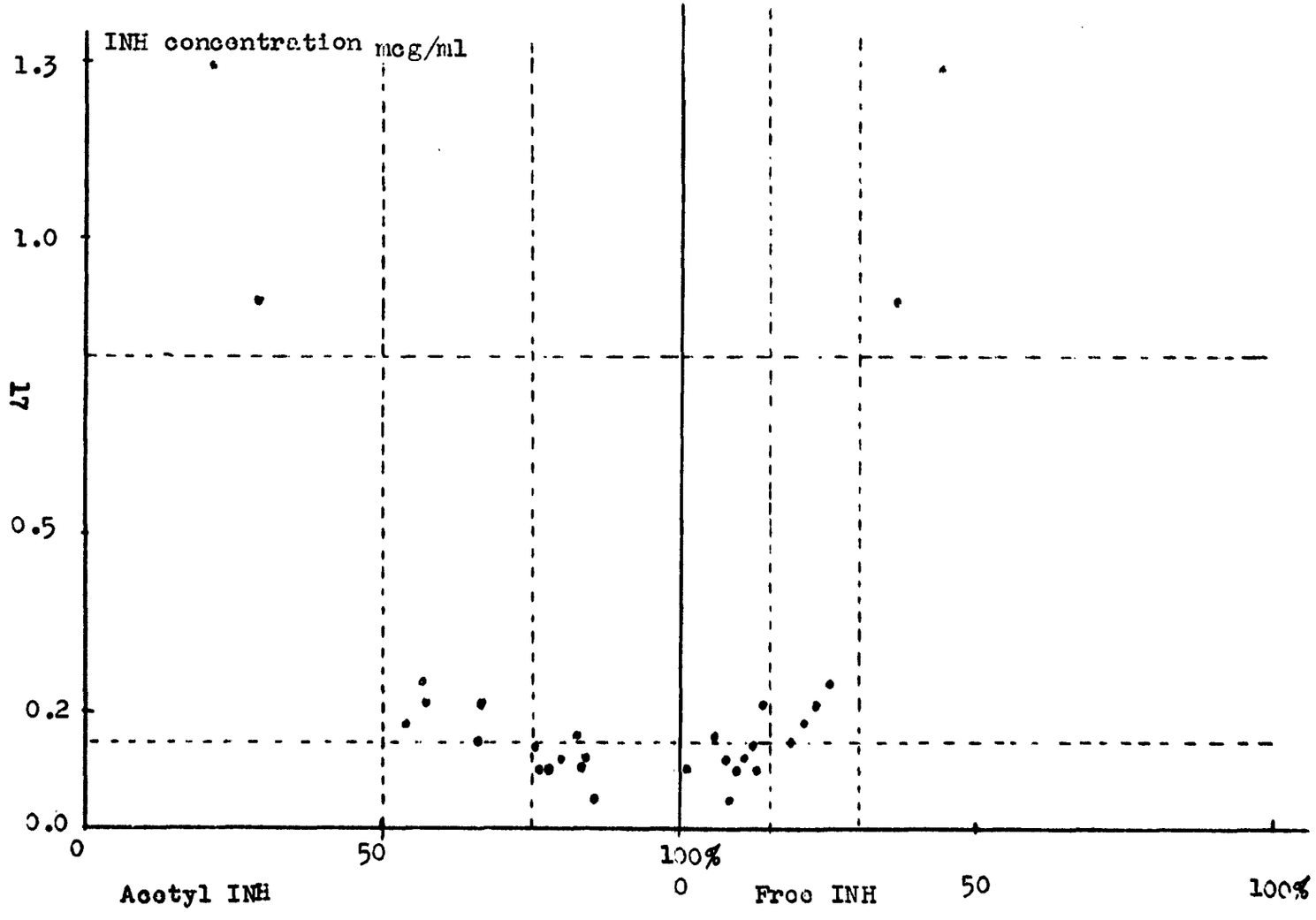


Fig. 15 Relationship between INH concentration in blood and acetyl and free INH in urine in 12 hours

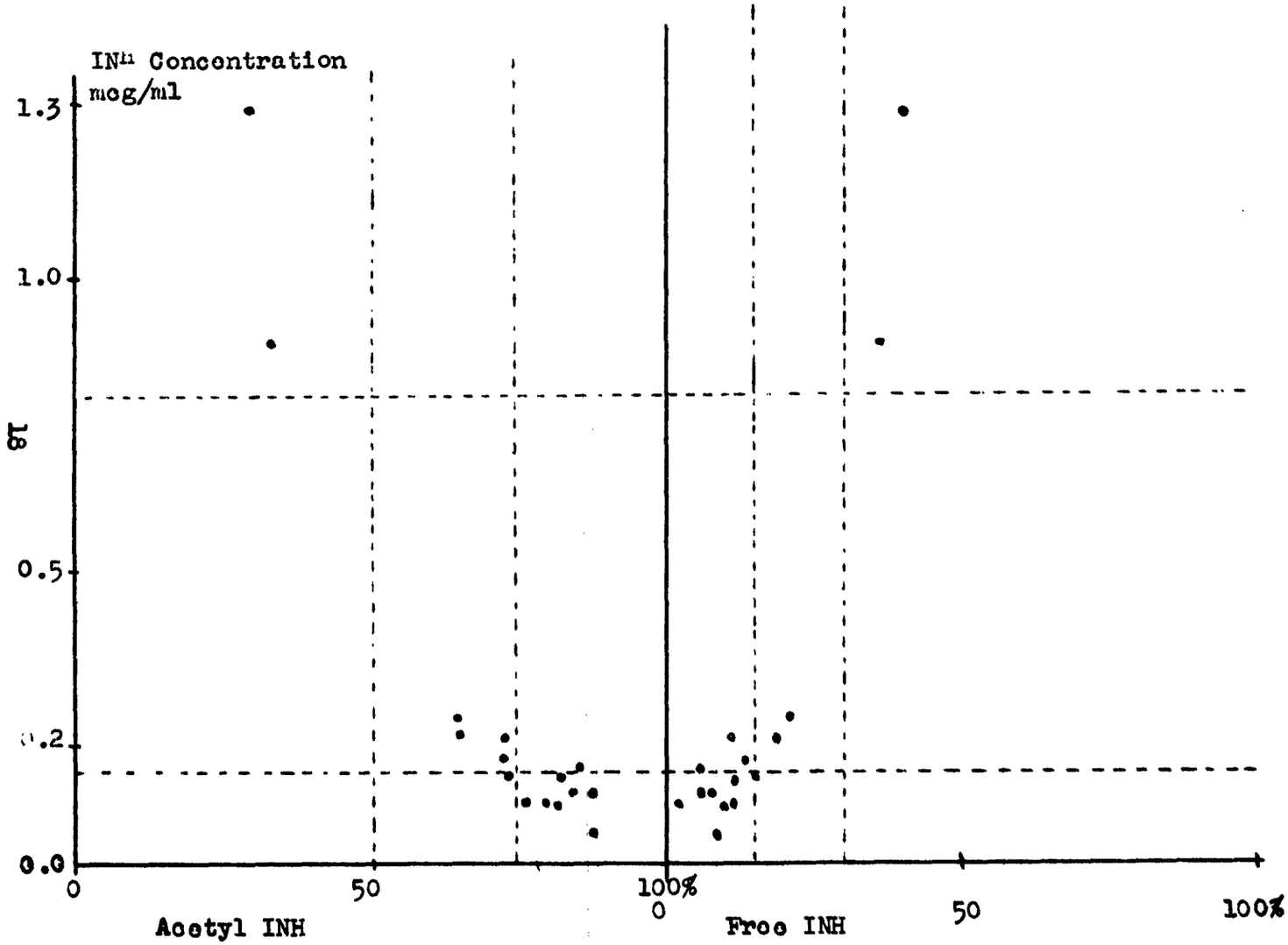
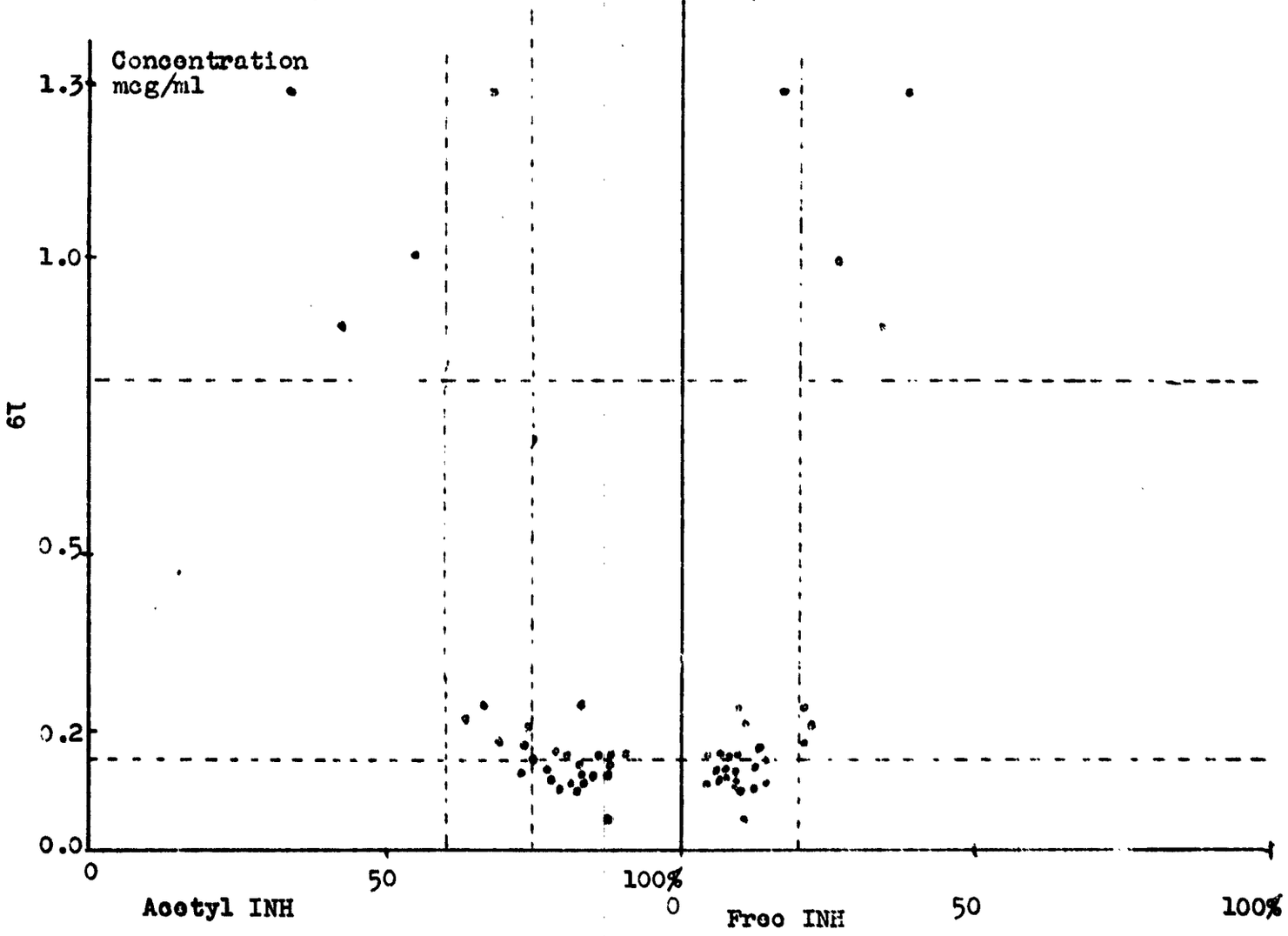


Fig. 16 Relationship between INH concentration in blood and acetyl and free INH in urine in 24 hours



IV STUDIES ON ISONIAZID INACTIVATION OF MONKEY

In view of establishing the relationship between blood levels of isoniazid and acetylating activity of the animal tissue and at the same time for the purpose of investigating the metabolic patterns of isoniazid in the animal species which is very close to human beings phylogenitically, our research on the monkey (tynomolgus—MACACA IRUS) is now in progress.

In this experiment, the reaction system for the monkey tissue homogenate is arranged so as to contain a sufficient amount of ATP and co-A as follows:

$\frac{M}{10}$ phosphate buffer (Ph 7.4)		0.5 ml
$\frac{M}{50}$ Na ATP		0.3
$\frac{M}{10}$ $MgCl_2$		0.1
$\frac{4}{10}$ M Na F		0.1
$\frac{M}{10}$ Na acetate		0.4
500 mcg/ml INH		0.4
Homogenate (25%)		1.0
1 mg/ml co-A		0.2
	Total	3.0
20% KOH		0.2

Incubated at 37° for 30 minutes

Fig. 17 indicates blood levels after the oral dose of 4 mg/kg body weight, 8 mg/kg body weight and 16 mg/kg body weight. As the blood levels after the dose of 4 mg/kg is too low, it seems necessary to take 8 or 16 mg/kg as a test dose. Though we intend to draw a frequency distribution curve of the blood levels of the monkey, the number of the animals is too small to draw a reliable curve for the time being.

Fig. 18 & Table 5 show the acetylating capacities of the tissue homogenates.

Fig. 17 Relationship of the blood levels 4 and 6 hours after administrations of 4, 8, 16 mg/kg INH to monkey

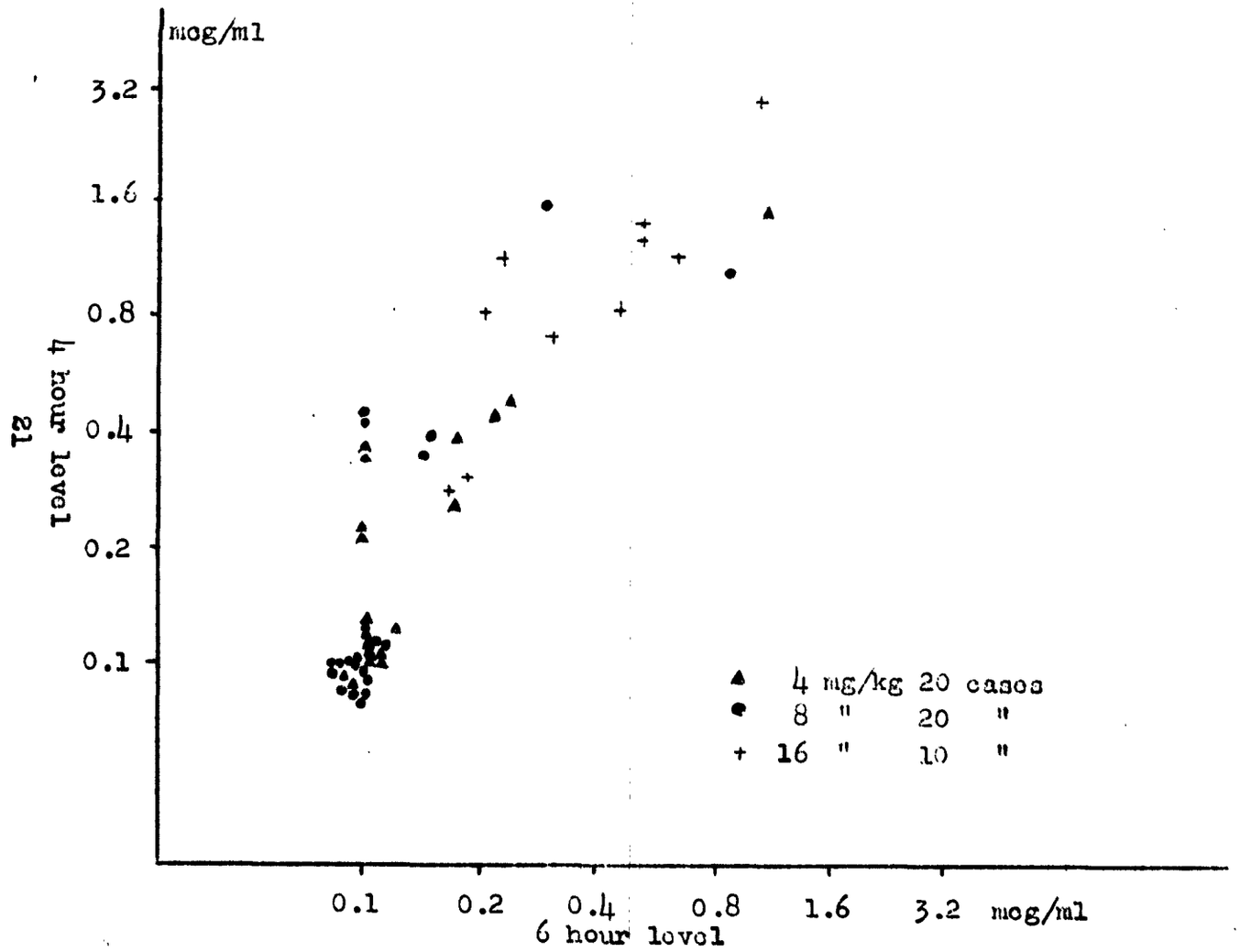


Fig. 18 Acetylating capacity of monkey liver (30 monkeys)

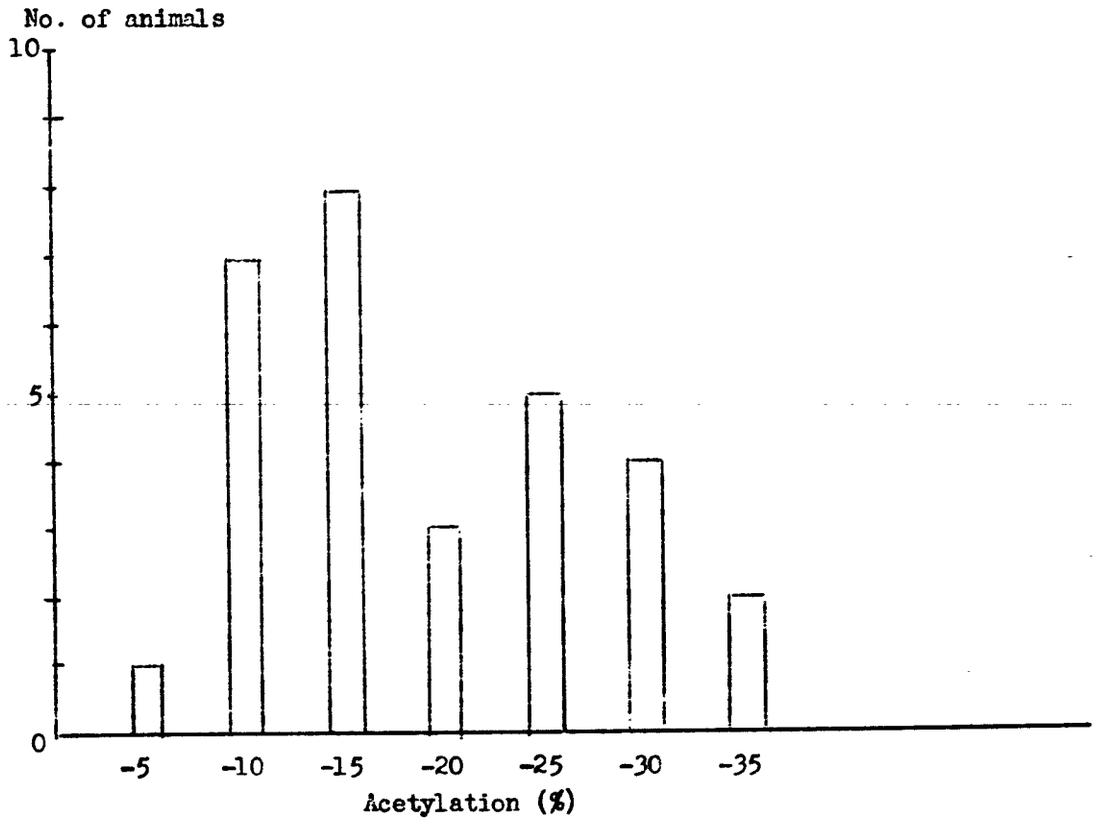


Table 5 Acetylating capacities of monkey tissue homogenates

Animal No.	Liver	Spleen
1	3.5%	5.6%
2	12.1	3.5
3	10.6	4.0
4	14.6	6.4
5	15.9	1.1
6	23.2	3.1
7	9.2	0
8	7.2	2.1
9	7.1	0
10	5.2	0.8
11	30.6	0
12	24.3	4.9
13	9.4	2.4
14	25.6	1.5
15	9.2	1.0
16	11.7	4.5
17	11.2	7.2
18	15.8	1.0
19	31.4	0
20	21.8	5.5
21	21.6	12.0
22	13.6	0
23	9.2	12.5
24	27.5	2.5
25	27.2	0
26	16.5	3.7
27	10.7	2.4
28	10.9	0
29	20.6	0
30	26.1	0
Average	16.12	2.94

Animal No. 11

Liver	30.6%
Kidney	12.0
Heart	0
Brain	0.5
Lung	0
Spleen	0

V FKI (FURYL-2-METHYL KETON ISONICOTINYLHYDRAZONE)

FKI is a new derivative which has been synthesized in the laboratory of the Daiichi Seiyaku Company in Tokyo in 1955 and later commercialized in Italy. Italian investigators reported that it showed a very low minimum inhibitory concentration against mycobacterium tuberculosis in vitro, but we could not confirm their findings. Prof. Kakkemi of Kyoto University studied the pattern of urinary excretion of free isoniazid after the dose of FKI to animals and supposed that this new compound must be highly long acting.

The result of our determination of the biologically active isoniazid level after the oral dose of FKI in comparison of isoniazid itself and other kinds of isoniazid which are commercialized in Japan is as follows:

Dose	INH	300mg	(Enteric coating)
	IHMS (INH-metasulfate)	300mg as INH	(" ")
	INHG (INH-glucuronate)	"	(" ")
	IPC (INH-ca-pyruvate)	"	(" ")
	FKI	"	(" ")

FKI showed higher levels than others at 10 hours as indicated in Fig. 19.

Fig. 19 Comparison of blood levels after the dose of various kinds of INH derivatives